JOURNAL OF VIROLOGY, Feb. 1975, p. 386-392

Vol. 15, No. 2
Printed in U.S.A.

Ribonucleoproteins of Uukuniemi Virus are Circular

RALF F. PETTERSSON* AND CARL-HENRIK VON BONSDORFF[1]
Department of Virology, University of Helsinki, 00290 Helsinki 29, Finland

Received for publication 6 September 1974

The internal ribonucleoprotein (RNP) of Uukuniemi virus was released with Triton X-100 and analyzed on sucrose gradients. Three species of RNP sedimenting at 140 to 150, 105 to 120, and 85 to 90*S* could be separated. All of them contained the same ratio of core polypeptide (mol wt, 25,000) to RNA. Electron microscopy using rotatory shadowing showed that all three species were circular. Free ends were rarely seen. Measurements of the strands revealed three distinct length classes of about 2.8, 1.4 and 0.7 μm. Polyacrylamide-agarose gels showed that the largest RNP contained the L RNA, the medium-sized RNP the M RNA, and the smallest RNP the S RNA.

Uukuniemi virus is the prototype of the Uukuniemi group of arboviruses (2, 14; J. Casals, personal communication) and represents a novel structural type of virus (16, 17, 20, 21, 24; C.-H. von Bonsdorff and R. F. Pettersson, manuscript in preparation). The spherical virus particle is about 90 to 100 nm in diameter (20) and has a lipid envelope (18) containing one or probably two polypeptide species (mol wt, 65,000 to 75,000) (17), which form a regular hexagonal surface lattice (20; von Bonsdorff and Pettersson, manuscript in preparation). The internal nucleoprotein consists of single-stranded RNA and multiple copies of a polypeptide with a molecular weight of about 25,000 (17). Previous results showed that at least four species of RNA can be isolated from purified virus preparations. The molecular weights of these were estimated to be about 4.1×10^6 (L), 1.9×10^6 (M), 0.88×10^6 (S1), and 0.78×10^6 (S2), whereas the sedimentation values in sucrose gradients were 27 to 29 (L), 22 (M), and 17 to 19*S* (S1 and S2) (ref. 16).

Pelleted virus particles stored at 4 C spontaneously release coiled strands about 9 nm wide and composed of a strand 2 nm thick (24). Similar strands, at least 1,000 nm long, have also been isolated from purified particles after treatment with detergent and have been considered to represent the nucleoprotein of Uukuniemi virus (17).

In this paper we have studied the size and form of Uukuniemi virus nucleoprotein using electron microscopy and biochemical methods.

MATERIALS AND METHODS

Cells. The origin and cultivation of chicken embryo cells (16, 22) and baby hamster kidney cells (BHK-21), clone WI-2 (17), have been described previously.

Virus. The stock virus used was prepared from the prototype strain S23 of Uukuniemi virus (14), red plaque variant, after five successive clonings in primary cultures of chicken embryo cells and two cultivation cycles in secondary chicken embryo cells (16).

Virus labeling and purification. Monolayers of secondary cultures of chicken embryo cells were infected at a multiplicity of infection of about 5 PFU/cell, as described previously (16). The virus was cultivated in the presence of Eagle minimum essential medium supplemented with 0.2% bovine serum albumin (Armour Pharmaceutical Co., England). Five hours postinfection the radioactive isotopes were added: 60 μCi of carrier-free [^{32}P]orthophosphate per ml (Institutt for Atomenergi, Kjeller, Norway) in minimum essential medium containing one-tenth of the normal amount of phosphate and, to two or three dishes per experiment, 35 μCi of [^{35}S]methionine per ml (129 Ci/mmol; The Radiochemical Centre, Amersham, England) in methionine-free minimum essential medium. The culture fluid was harvested 20 h postinfection, and the radioactive virus was concentrated and purified as described previously (16, 17), but with some modifications. The first pelleting step was carried out by centrifuging the virus through a 3-ml cushion of 15% (wt/wt) sucrose made in 0.1 M NaCl and 0.05 M Tris, pH 7.4 (TN buffer). Treatment of the pelleted virus with DNase (16) was omitted. The final pelleting step was carried out by centrifugation at 24,000 rpm for 90 min at 4 C in an SW27 rotor.

Isolation of RNP and analysis on sucrose gradients. The [^{32}P]- and [^{35}S]methionine-labeled nucleoprotein was released from whole virus by treatment with 1% Triton X-100 for 20 min at 4 C (17). The ribonucleoprotein (RNP) was analyzed on 15 to 30% (wt/wt) sucrose gradients made in TN buffer containing 0.05% Triton X-100 (wt/vol). In some experiments the detergent was omitted from the gradient. Centrifugation was for 5, 5.5, or 6 h at 24,000 rpm and 4 C in a Spinco 27 rotor using narrow tubes. [^{35}S]methionine-labeled encephalomyocarditis virus (160*S*; ref. 7), prepared as described for poliovirus (8), or Semliki Forest virus nucleocapsid (140*S*; ref. 8)

[1] Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass. 02318.

centrifuged in separate tubes were used as references. Sedimentation coefficients were estimated according to Martin and Ames (11).

Polyacrylamide-agarose gel electrophoresis of RNA. The virus RNAs released from the RNPs by treatment with 1 or 2% sodium dodecyl sulfate were analyzed on 2% polyacrylamide gels containing 0.5% agarose and 0.1% sodium-dodecyl sulfate, according to Peacock and Dingman (15), and as described previously (16).

SDS-polyacrylamide gel electrophoresis of proteins. [^{35}S]methionine-labeled viral proteins were analyzed on 7.5% gels according to Weber and Osborn (25) and as described previously (17).

Radioactivity assay. Samples to be analyzed were precipitated with ice-cold trichloroacetic acid, collected on membrane filters (Millipore Corp.), dried, and counted as described previously (17).

Electron microscopy. Samples for electron microscopy were taken from the peak fractions or from pooled fractions of the sucrose gradients, either as such or after dialysis overnight against 1% ammonium acetate, pH 7.0. A drop of the sample was applied to a Formvar-coated copper grid, and the excess liquid was removed with filter paper. The grids with sucrose-containing specimens were washed briefly with distilled water before staining negatively with 2% potassium phosphotungstate, pH 7.0.

Rotatory shadowing. A Balzers BA 360M freeze-etching unit equipped with a rotatory shadowing device was used for this purpose. The grids were mounted on the specimen holder and rotated at 50 rpm during shadow-casting with Pt (95%)-C (5%) at an angle of 10°. The specimens were examined in a Siemens Elmiskop IA electron microscope, and micrographs were taken at original magnifications of 20,000 to 40,000 calibrated with a carbon grating of 54,865 lines/inch (Ladd Industries, Inc., Burlington, Vt.). Length measurements were made from micrographs at a final magnification of 120,000 times with the aid of a map measurer.

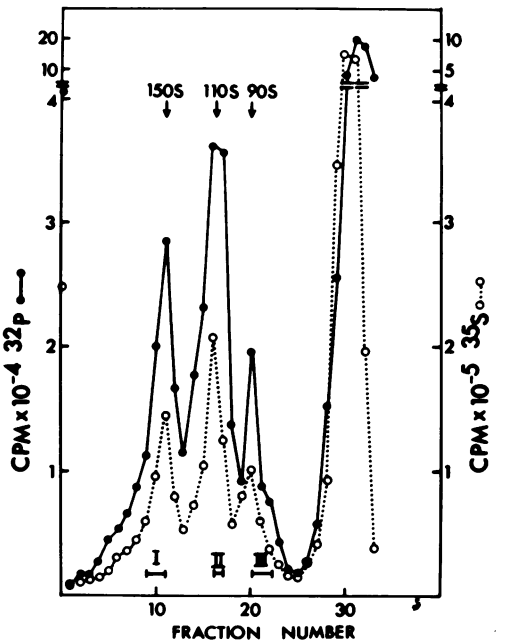

FIG. 1. *Fractionation of Uukuniemi virus nucleoproteins. Purified Uukuniemi virus double labeled with [^{32}P]- and [^{35}S]methionine was treated with 1% Triton X-100 for 20 min at 4 C and analyzed on a 15 to 30% (wt/wt) sucrose gradient made in TN buffer. Centrifugation was carried out at 24,000 rpm for 6 h at 4 C in a Spinco SW27 rotor. [^{35}S]methionine-labeled Semliki Forest virus nucelocapsid (140S) and encephalomyocarditis virus (160S) were centrifuged in separate tubes as references. Fractions (0.5 ml) were collected from below, and samples were analyzed for trichloroacetic acid-precipitable radioactivity. Bottom at left, zero fraction represents the pellet. Symbols: [^{32}P], ●, [^{35}S], O.*

RESULTS

Sedimentation of RNP in sucrose gradients. Uukuniemi virus RNP was released from whole virus with 1% Triton X-100 (17), and the product was analyzed on sucrose gradients. Figure 1 shows the sedimentation pattern obtained in an experiment where the virus was labeled with [^{32}P]- and [^{35}S]methionine. In addition to the envelope fraction on top of the gradient, three peaks of radioactivity containing both labels can be identified. These peaks had a similar ratio of protein-RNA label (range 4.5 to 5.6) and sedimentation values of about 150, 120, and 90S, as calculated from three experiments. Resedimentation of the peak fractions in 15 to 30% sucrose gradients containing 0.05% Triton X-100 resulted in a slight decrease in the sedimentation rates to about 140, 105, and 85S, respectively.

The peak fractions were pooled separately (Pool I to III in Fig. 1) and analyzed on polyacrylamide gels for their RNA (Fig. 2) and protein (data not shown) composition. Pool I contained predominantly the L RNA, much M RNA, and little S RNA. The molar ratios (L-M-S) in this experiment were about 5:2.5:1, as compared with a ratio of 1:4.6:1 normally found in preparations of whole virus (16). Pool II contained almost exclusively the M RNA (1:112:5.5). Pool III consisted mainly of S RNA but was contaminated with both M and L RNAs (1:9:40). In the 2.0% polyacrylamide gels used in this experiment the two small S1 and S2 RNAs (16) were not separated from each other.

All three peaks contained only the core polypeptide with an apparent molecular weight of 25,000 daltons (17), whereas the top fractions contained the envelope protein(s) (17), together with the ^{32}P-labeled phospholipids (18). No hemagglutinating activity (19) was found in the three peaks (fractions 1 to 25 of Fig. 1). The top fractions contained all the hemagglutinating

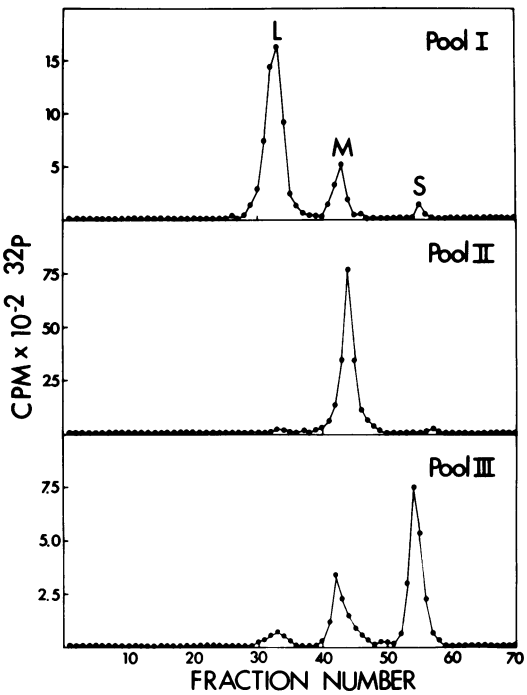

FIG. 2. *Separation of Uukuniemi virus L, M, and S RNAs on polyacrylamide-agarose gels. Samples from pools I, II, and III of Fig. 1 were treated with 2% sodium dodecyl sulfate and analyzed on 2.0% polyacrylamide gels containing 0.5% agarose and 0.1% sodium dodecyl sulfate. The gels were run at 10 V/cm for 3 h at room temperature and then cut into 2-mm slices and counted in NCS-toluene. Migration is from left to right.*

activity recovered, indicating that the envelope protein(s) is responsible for the hemagglutinating property. The three nucleoprotein species are hereafter referred to as the L (large), M (medium), and S (small) RNP.

Electron microscopy of the RNPs. Negative staining of the RNP pools with potassium phosphotungstic acid at neutral pH showed an abundance of strands, which were about 2 nm thick and similar to those described earlier (17). Since individual strands were rarely resolved over their whole length, it was impossible to obtain reliable size determinations with this method. However, a rotatory shadowing technique was suitable for this purpose. The RNP material shown in Fig. 3A was taken directly from the sucrose gradient (pool I of Fig. 1) and treated as described in Materials and Methods. In such preparations rather few of the RNP strands were extended to their full length. Those which were stretched out appeared in all cases to be composed of two more or less parallel strands, which were in many instances partly separated from each other. Better uncoiling and separation of the individual strands was obtained when samples were dialyzed against 1% ammonium acetate prior to preparation for electron microscopy (Fig. 3B and C; Fig. 4A-C).

In almost all cases in which the strands could be followed over their whole length, the three RNP species were seen as closed circles (Fig. 3C; Fig. 4A-C). Only rarely were free ends found (Fig. 3B, arrows).

For length measurements only fully extended strands, like those shown in Fig. 4A to C, were selected. The strands fall into three distinct size classes irrespective of the RNP pool from which they were recovered (Fig. 5). The largest had a mean length of 2.77 ± 0.10 (standard deviation) μm (L RNP), the medium-sized 1.41 ± 0.07 μm (M RNP), and the smallest 0.75 ± 0.06 μm (S RNP). Separation of the strands according to their size was not complete in the sucrose gradient. As judged from the length determinations, pool I contained, in addition to the L RNP strands, some M and S RNP strands. Pool II contained almost exclusively M RNP strands, whereas in pool III all species were commonly found.

DISCUSSION

The present results show that RNP strands of at least three different lengths can be isolated from purified Uukuniemi virus preparations. The strands, which have different sedimentation properties in sucrose gradients and thus can be separated, fall into three distinct size classes of about 2.8, 1.4, and 0.7 μm. As judged from electron micrographs of RNPs visualized by rotatory shadowing, all three species are closed circles. This finding is in conformity with previous observations that no free ends of the RNP strands released either from spontaneously disintegrating (24) or detergent disrupted particles (17) were detected. It therefore seems likely that the Uukuniemi virus RNP strands occur as circular rather than linear structures.

As determined from the [^{32}P]-[^{35}S] ratio, the RNP species seem to have an identical RNA-protein ratio. This agrees with previous results, showing that the RNPs band as a single peak in CsCl (ρ = 1.31 g/cm^3) (17).

Although the molar ratio of the RNA species in each RNP pool (Fig. 1) could be calculated, the relative amount of the three RNP species in the pools could not be accurately estimated. This was due to the fact that in all preparations a considerable portion of the strands were coiled, making length measurements impossible. The results, however, suggest that the largest L RNP contains the L RNA, the inter-

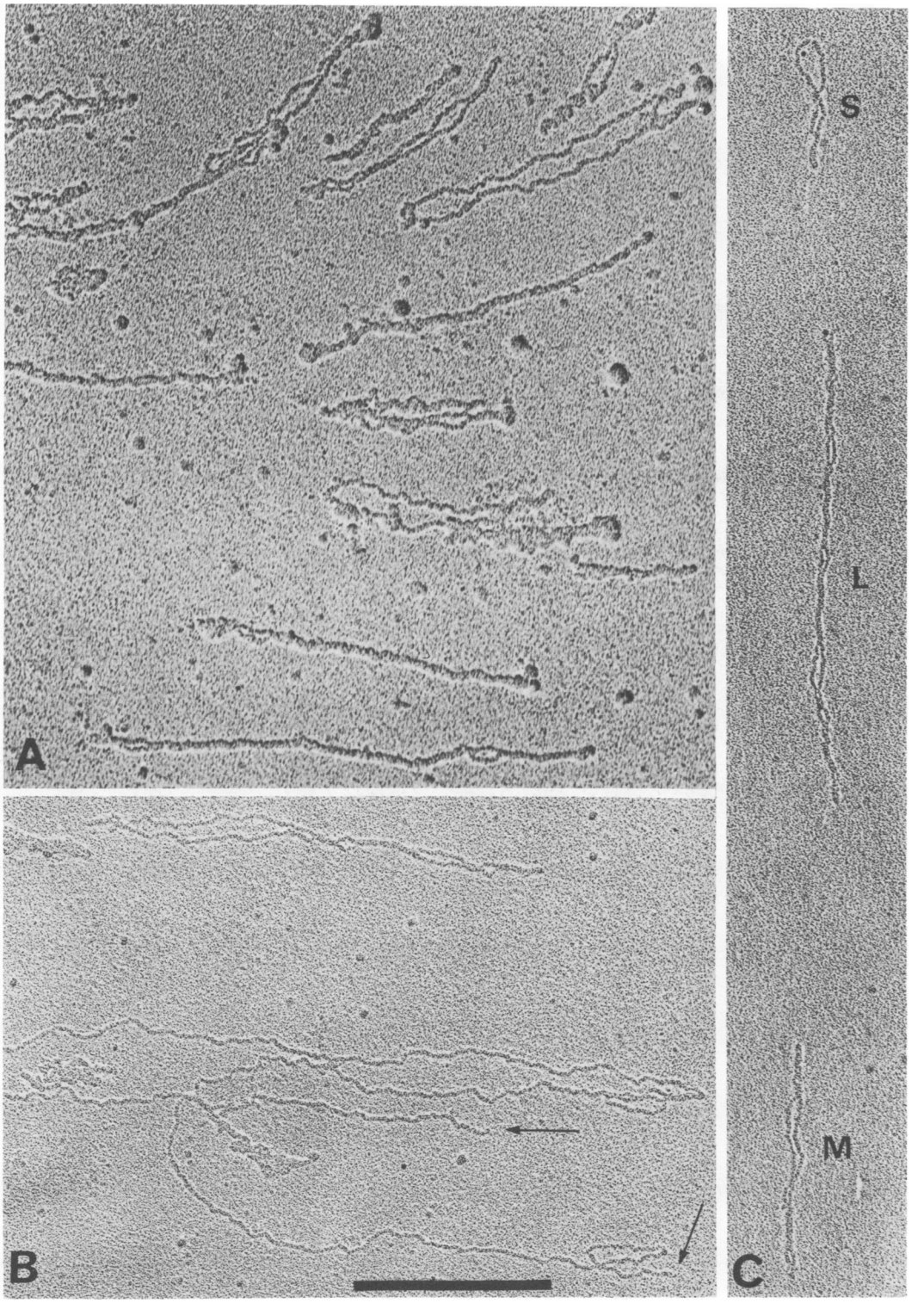

FIG. 3. *Electron micrographs of Uukuniemi virus RNPs. Samples were taken from pool I (Fig. 1) and prepared for electron microscopy either without (A) or with (B, C) dialysis against 1% ammonium acetate. The RNPs were visualized by rotatory shadowing. Arrows, "free ends" of RNP. (C) All three RNPs species shown in the same field. Bar, 0.5 μm.*

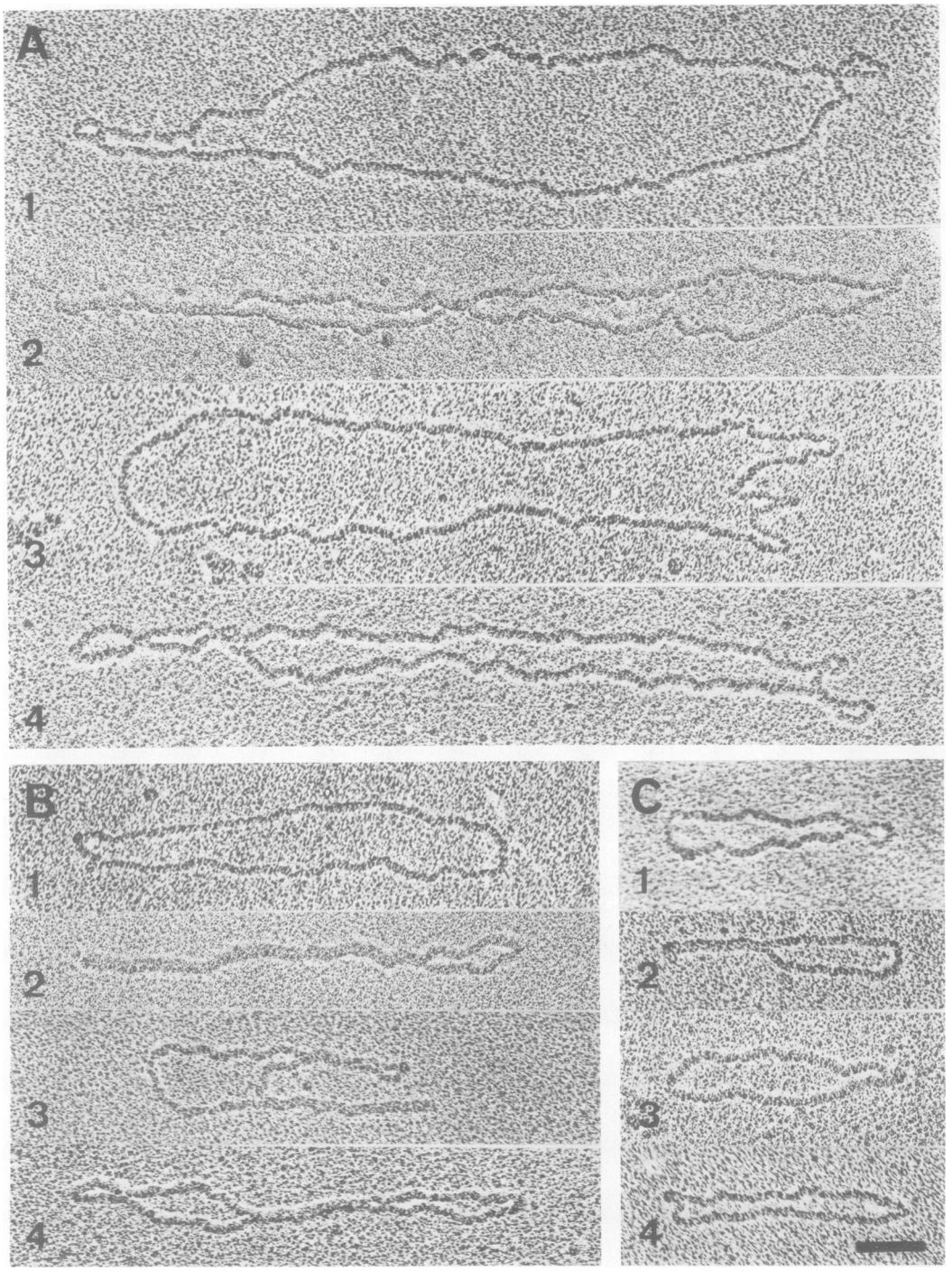

FIG. 4. *Electron micrographs of the circular RNPs of Uukuniemi virus. Examples of fully extended strands from the RNP pools I, II, and III (Fig. 1) selected for length measurements. (A) L RNP; (B) M RNP; and (C) S RNP. Bar, 100 nm.*

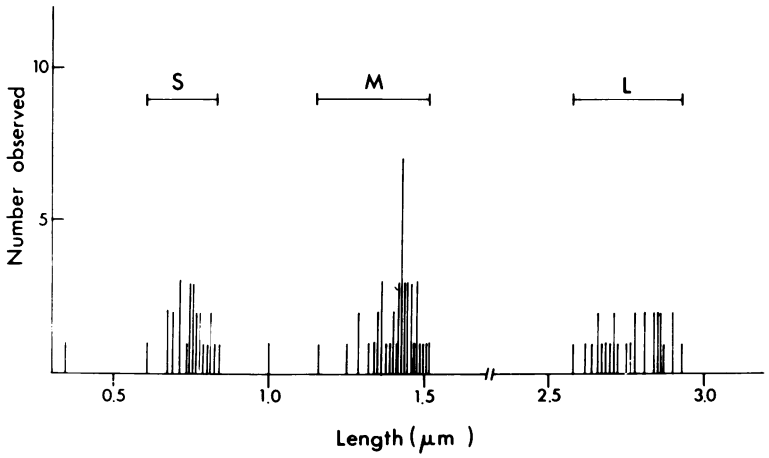

FIG. 5. *Length distribution of Uukuniemi virus RNPs. A total of 97 circular strands obtained from pools I, II, and III (Fig. 1) were analyzed.* ⊢—⊣, *Size range of each RNP class selected for determination of the mean length.*

mediate M RNP the M RNA, and the smallest S RNP the S RNA. From the apparent molecular weights of L and M RNAs one would expect RNPs with lengths close to 4 and 2 μm, respectively, assuming both a linear RNA in the RNPs, and that 1 μm approximately corresponds to 10^6 daltons of RNA (6, 10). However, RNPs of lengths only 2.8 and 1.4 μm were observed. For the S RNP a better correlation between the measured RNP length and the estimated molecular weight of the RNA was obtained.

An important question is how many of each RNP species are packed in the virions. The RNA species isolated from virus particles grown in chicken embryo cells have not been found to occur in equimolar ratio, but rather in a molar ratio of 1:4.6:1:1 (L-M-S1-S2) (16). In the light of this finding the possibility that the RNA species are present in any one particle in their molar ratio seemed improbable, although not impossible (16). The space inside the viral lipid layer is roughly 1.5×10^5 nm^3. On the basis of available data (16, 17) one can calculate that this is more than sufficient to enclose one L RNP, four to five M RNP, and two S RNPs (containing one S1 and one S2, respectively). Thus, both the quantity and quality of the RNP species inside each virion still remain obscure.

Different species of RNP have also been isolated from vesicular stomatitis virus (9) and influenza virus (4), both of which are enveloped RNA viruses. The three species of vesicular stomatitis virus RNP strands, which occur in the B, LT, and T particles (9), differ from Uukuniemi virus RNP in being typical helixes (13). Influenza virus RNP also falls into distinct size classes. All the RNP strands are contained in the same virus particle, each strand being folded back on itself to form a twisted double helix (4, 23). In neither of these viruses have the RNP strands been found to occur in a circular form, and the linear helical nucleoprotein of Newcastle disease virus has been reported to occur as a circular structure in only less than 1% of the strands obtained from the cytoplasm of Newcastle disease virus-infected cells (3). Since no circular RNPs have been found in any other RNA-containing virus, Uukuniemi virus RNP apparently represents a novel structure.

The small bacteriophages ΦX174 (5) and fd (12) have been shown to contain circular single-stranded DNA. No such structure has been reported for viruses possessing a genome of single-stranded RNA. Whether the presence of circular RNP strands in Uukuniemi virus means that the RNAs are also circular remains to be established.

ACKNOWLEDGMENTS

We wish to thank Annikki Kallio, Kaija Kettunen, and Anssi Mörttinen for skillful assistance and Niall Glanville for correcting the language.

This investigation was supported by the Sigrid Jusélius Foundation, The National Research Council for Medical Sciences, Finland, and by the Finnish Medical Association (Finska Läkarasällskapet).

LITERATURE CITED

1. Burness, A. T. H., A. D. Vizoso, and F. W. Clothier. 1963. Encephalomyocarditis virus and its ribonucleic acid: sedimentation characteristics. Nature (London) **197**:1177-78.
2. Casals, J. 1967. Antigenic characterization of tick-borne arboviruses not belonging in group B. Jpn. J. Med. Sci. Biol. **20** (suppl.):119-129.
3. Compans, R. W., and P. W. Choppin. 1967. The length of

the helical nucleocapsid of Newcastle Disease virus. Virology **33**:344-346.
4. Compans, R. W., J. Content, and P. H. Duesberg. 1972. Structure of the ribonucleoprotein of influenza virus. J. Virol. **10**:795-800.
5. Fiers, W., and R. L. Sinsheimer. 1962. The structure of the DNA of bacteriophage ΦX174. III. Ultracentrifugal evidence for a ring structure. J. Mol. Biol. **5**:424-34.
6. Granboulan, N., and M. Girard. 1969. Molecular weight of poliovirus ribonucleic acid. J. Virol. **4**:475-479.
7. Kääriäinen, L., K. Simons, and C.-H. von Bonsdorff. 1969. Studies in subviral components of Semliki Forest virus. Ann. Med. Exp. Biol. Fenn. **47**:235-248.
8. Kääriäinen, L., and H. Söderlund. 1971. Properties of Semliki Forest virus nucleocapsid. I. Sensitivity to pancreatic ribonuclease. Virology **43**:291-299.
9. Kiley, M. P., and R. R. Wagner. 1972. Ribonucleic acid species of intracellular nucleocapsids and released virions of vesicular stomatitis virus. J. Virol. **10**:244-255.
10. Kolakovsky, D., E. Boy de Tour, and H. Delius. 1974. Molecular weight determination of Sendai and Newcastle disease virus RNA. J. Virol. **13**:261-268.
11. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372-1379.
12. Marvin, D. A., and H. Schaller. 1966. The topology of DNA from the small filamentous bacteriophage fd. J. Mol. Biol. **15**:1-7.
13. Nakai, T., and A. F. Howatson. 1968. The fine structure of vesicular stomatitis virus. Virology **35**:268-281.
14. Oker-Blom, N., A. Salminen, M. Brummer-Korvenkontio, L. Kääriäinen, and P. Weckström. 1964. Isolation of some viruses other than typical tick-borne encephalitis viruses from Ixodes ricinus ticks in Finland. Ann. Med. Exp. Biol. Fenn. **42**:109-112.
15. Peacock, A. C., and C. W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry **7**:668-674.
16. Pettersson, R., and L. Kääriäinen. 1973. The ribonucleic acids of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **56**:608-619.
17. Pettersson, R., L. Kääriäinen, C.-H. von Bonsdorff, and N. Oker-Blom. 1971. Structural components of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **46**:721-729.
18. Renkonen, O., L. Kääriäinen, R. Pettersson, and N. Oker-Blom. 1972. The phospholipids of Uukuniemi virus, a non-cubical tick-borne arbovirus. Virology **50**:899-901.
19. Saikku, P. 1974. Passerine birds in the ecology of Uukuniemi virus. Med. Biol. **52**:98-103.
20. Saikku, P., and C.-H. von Bonsdorff. 1968. Electron microscopy of the Uukuniemi virus, an ungrouped arbovirus. Virology **34**:804-806.
21. Saikku, P., C.-H. von Bonsdorff, and N. Oker-Blom. 1970. The structure of Uukuniemi virus. Acta Virol. **14**:103-107.
22. Sandelin, K., and T. Estola. 1970. Studies on the possibility of obtaining leukosis-free eggs from commercial poultry flocks with reference to current laboratory methods. Acta Pathol. Microbiol. Scand. Sect. B **78**:473-478.
23. Schultze, I. T. 1972. The structure of influenza virus. II. A model based on the morphology and composition of subviral particles. Virology **47**:181-196.
24. von Bonsdorff, C.-H., P. Saikku, and N. Oker-Blom. 1969. The inner structure of Uukuniemi virus and two Bunyamwera supergroup arboviruses. Virology **39**:342-344.
25. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406-4412.